Research Article

90-Days Repeated Dose Oral Toxicity Study on a Traditional Polyherbal Formulation Used in Urinary Disorders

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ABSTRACT

Majoon-e-Kundur (MK) is a compound Unani Pharmacopoeial formulation used in Taqteer-ul-Baul (Dribbling of urine), Salas-ul-Baul (Urinary incontinence), BaulFilfarash (Nocturnal enuresis), Surat-e-Inzal (Premature ejaculation) and Zof-e-Masana (Weakness of urinary bladder). Present study is designed to evaluate sub-chronic toxicity of MK in rats. Present study was carried out to evaluate 90 days repeated dose oral toxicity of MK in rats.Study was carried out on SD rats of both sexes. Animals were divided into three groups (n=10 per sex per group). MK was administered at the dose of 1028 and 2000 mg/kgbw/day p.o. for 90 days. Control animals were administered with vehicle. After completion of 90-days, blood samples were collected for haematological and biochemical analysis and animals were sacrificed, organs were harvested for weight determination and histopathological evaluation. Animals in MK-treated groups did not reveal any abnormal behaviour or clinical signs indicative of systemic toxicity. There was no toxicologically significant alteration observed in body weight, feed intake, haematological and biochemical parameters, and relative organ weights of control and MK treated rats of either sex.No toxicologically significant effects were observed with respect to clinical signs of toxicity, haematology, clinical chemistry, organ weight and gross necropsy findings in MK treated rats at 1028 and 2000 mg/kg bw or in control animals. The histopathological finding did not reveal any toxicologically significant change. Thus, the No Observed Adverse Effect Level (NOAEL) of MK may be considered >2000 mg/kg bw in rats.

Keywords: Toxicity, Herbal, Majoon-e-Kundor, Unani, Kundur

INTRODUCTION

Majoon-e-Kundur (MK) is a compound Unani Pharmacopoeial formulation mentioned in the National Formulary of Unani Medicine, PART-I [1] and other classical text of Unani system of medicine. It is recommended for the treatment of Taqteer-ul-Baul (Dribbling of urine), Salas-ul-Baul (Urinary incontinence), Baul Filfarash (Nocturnal enuresis), Surat-e-Inzal (Premature ejaculation) and Zof-e-Masana (Weakness of urinary bladder). MK is a polyherbal Unani formulation which contains Juft Baloot (*Quercus incana*), Zanjabeel (*Zingiber officinale*), Saad Kufi (*Cyperus rotundus*), Filfil Siyah (*Piper nigrum*), Qust Shirin (*Saussurea lappa*), Kundur (*Boswellia serrata*). Though this formulation is being used clinically from decades, no data is documented for its long term safety following repeated use. Therefore, the present study was carried out to evaluate repeated dose oral toxicity of MK in rats in order to generate evidence for safety of this valuable Unani formulation and promote this formulation globally.

Materials And Methods

Experimental Animals

Sprague Dawley rats (5-6 weeks of age) were used for the study. Rats were obtained from National Institute of Nutrition, Hyderabad, India. The selected females were nulliparous and non-pregnant. Rats were housed in polycarbonate cages in the air conditioned room maintained at the temperature of 22°C ± 3°C and relative humidity of 30-70%, with a 12:12 h light/dark illumination cycle [2]. Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Guidelines of laboratory animal care were followed throughout the experiment [3]. Protocol of the study was approved by the Institutional Animals Ethics Committee wide protocol no. CRIUM/IAEC/2015/01/P01. Animals were provided with standard feed pellets (SAFE diet, France) and water *ad libitum*, unless stated otherwise. Animals were acclimatized to the laboratory conditions for one week before using them for experiment.

Drug / Formulation

MK used in present study was prepared as per the standard Pharmacopoeial procedure [1] in the GMP certified pharmacy of CRIUM Hyderabad.

Dose Selection

Therapeutic dose of Majoon-e-Kundor is reported as 5-10g per day [1]. For 90-day repeated dose oral toxicity on MK, two dose groups were included i.e., Therapeutic Equivalent Dose of 10 g human dose based on body surface area conversion (equivalent dose in rats is 1,028 mg/kg bw per day) [4] and approximately twice of TED i.e., 2,000 mg/kg bw per day. Third dose group with higher dose of MK is not feasible due to high clinical dose which is not justified in view of limit dose of 2,000 mg/kg as per ICH guideline [5].

Drug Administration

MK was administered as aqueous suspension in 0.3% CMC (<2mL/100 gm bw). Suspension was freshly prepared every day. The control animals were administered vehicle only. Doses were administered by oral gavage once daily for 90 consecutive days at similar time each day to minimize variations.

Duration of Treatment

Duration of toxicity study for MK was 03 months (i.e., 90-days).

Experimental Design

The 90-day repeated dose oral toxicity study was performed according to the OECD test guideline-408 [2]. Male and female Sprague Dawley rats were divided in to 3 groups with 20 animals (10 males + 10 females) in each group. First group served as vehicle control and was orally administered 0.3% aqueous CMC suspension daily for 90-days. Second and third group were given MK at the dose levels of 1028 and 2000 mg/kg bw per day p.o. for 90 days.

All the experimental animals were observed for mortality and morbidity twice a day, throughout the study duration. Detailed clinical observations (i.e., functional observation parameters) were made periodically to detect signs of toxicity, at the same time (1h after vehicle or drug administration). Body weight of the animals was recorded once in a week. Average feed intake for both sexes were recorded at weekly interval by weighing the amounts of feed given to a cage group and leftovers on the next day. At the end of the treatment period, the overnight fasted (water provided *ad-libitum*) rats were anaesthetized with isofluorane inhalation (EZ Anaesthesia-1339), blood samples were collected by retro-orbital puncture in the EDTA vacutainers (for haematological) and serum vacutainers (for biochemical and electrolyte analysis) as reported earlier [6]. Haemoglobin (Hb), red blood cell count (RBC), white blood cell count (WBC), haematocrit (HCT) and platelet (PLT) were analysed using fully automated haematology analyser (Swelab Autocounter-920EO+). Serum biochemical parameters such as glucose, Serum glutamic pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), total bilirubin, creatinine, blood urea nitrogen (BUN), total cholesterol (TC), triglycerides (TG), total protein (TP) and albumin were analysed using fully automatic analyser (Erba-EM200). Serum electrolytes such as sodium, potassium, chloride and total calcium were estimated in a fully automated electrolyte analyser (Allcare-AC9801). At the end of study duration, all animals were subjected to gross necropsy. Organs and tissues were examined macroscopically and internal organs / tissues were isolated, trimmed and weighed. Organs / tissues were preserved in the neutral buffered formalin and subjected to histological examination.

Statistical Analyses

Data expressed as mean ± standard error of mean (SEM). Mean difference between the control and treatment group was analysed by ANOVA using GraphPad prism (version 5). *P* value ≤ 0.05 was considered as statistically significant.

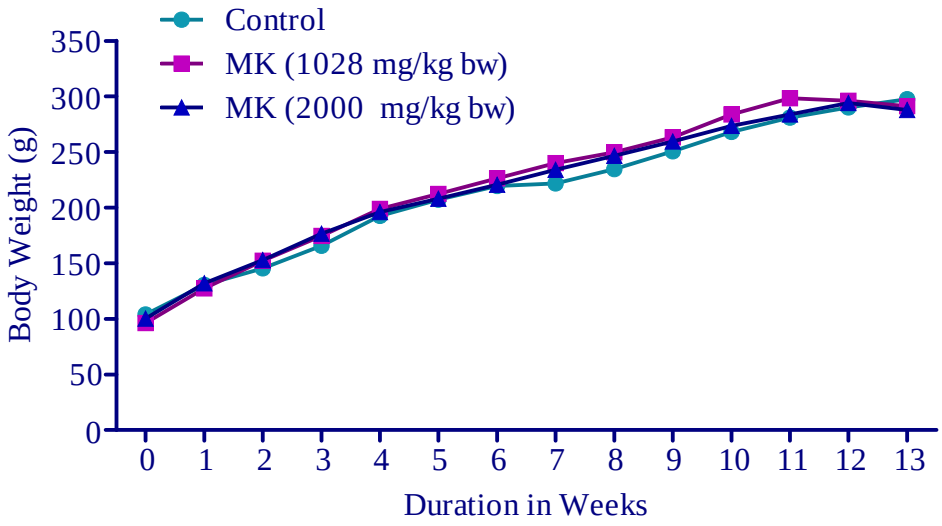


Figure 1(A): Average body weight of control and MK treated male rats

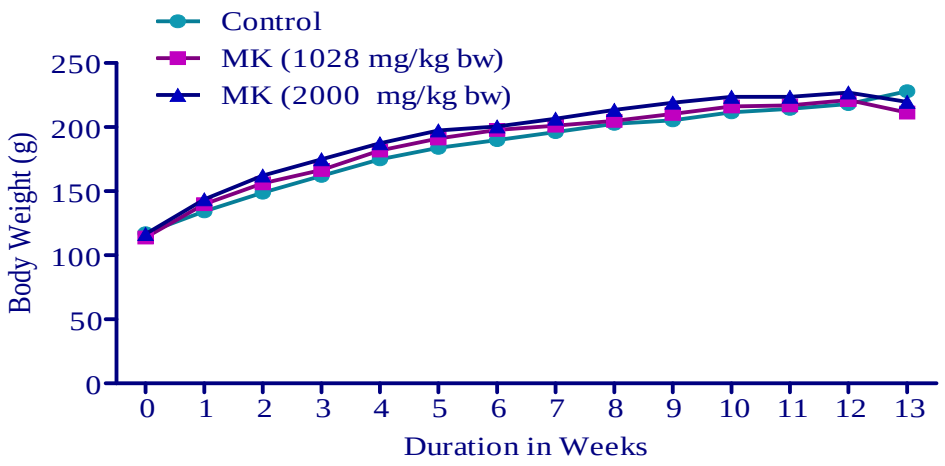


Figure 1(B): Average body weight of control and MK treated female rats

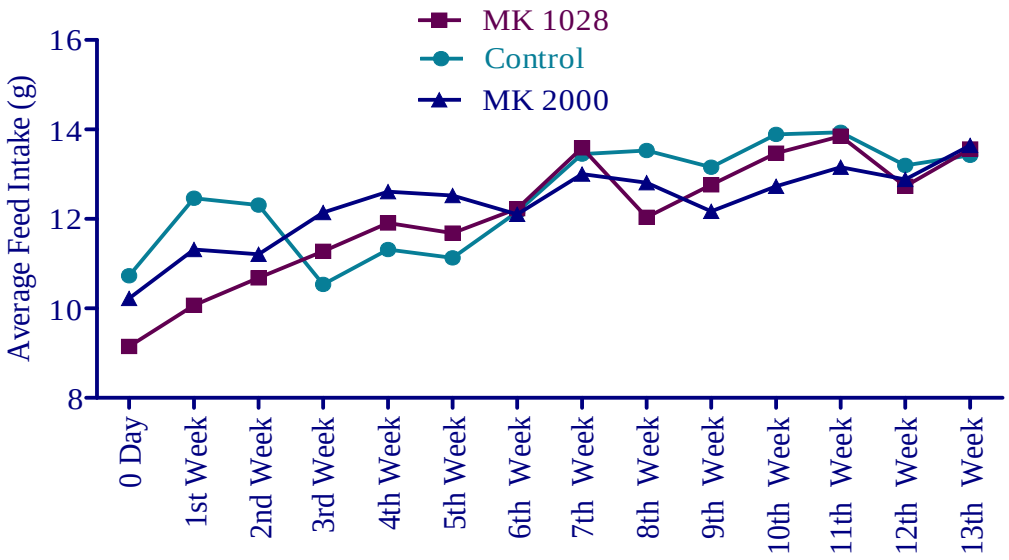


Figure 2 (A): Average feed intake of control and MK treated female rats

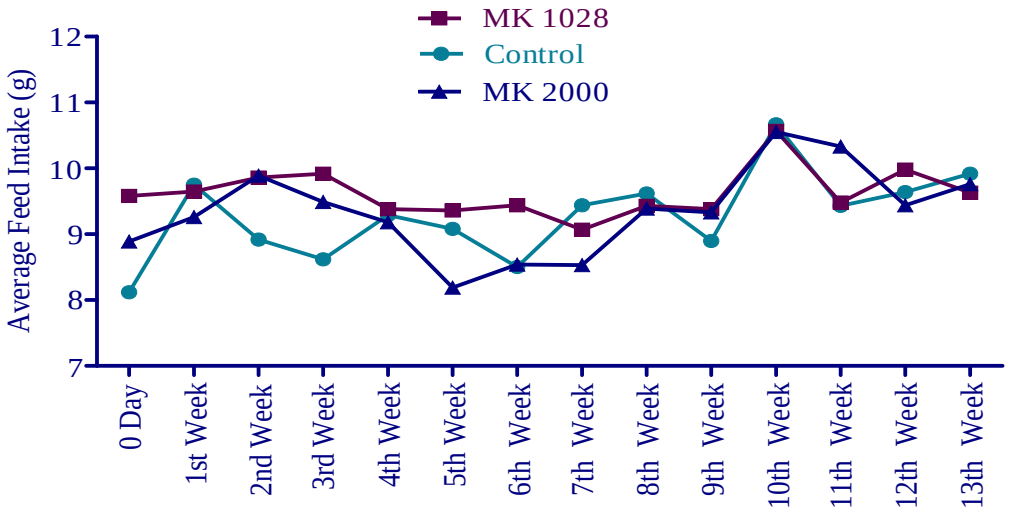


Figure 2 (B): Average feed intake of control and MK treated male rats

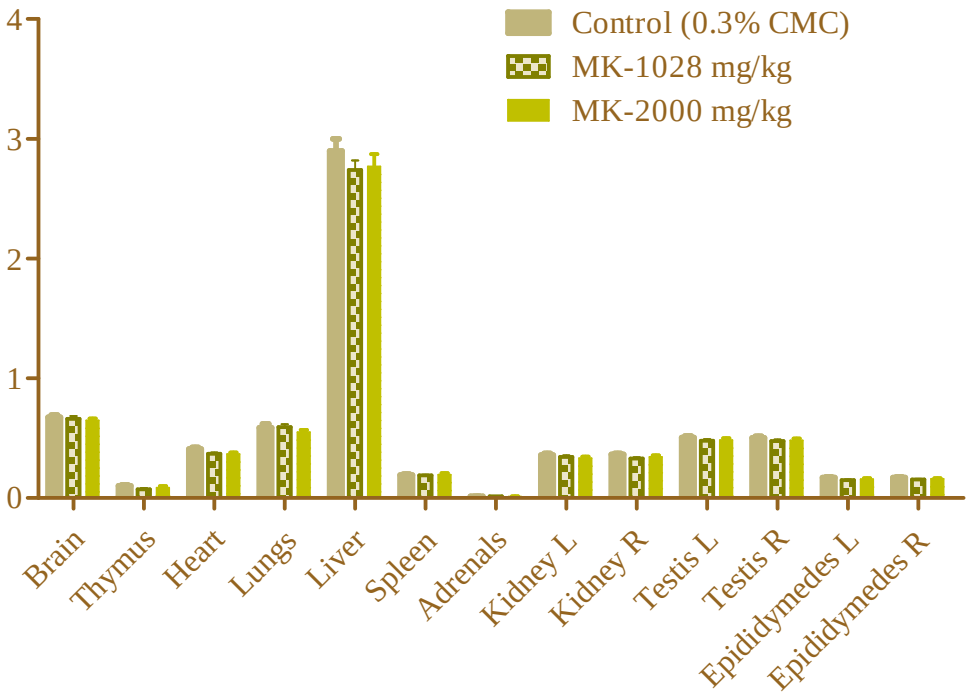


Figure 3 (A): Effect of MK on relative organ weights of male rats

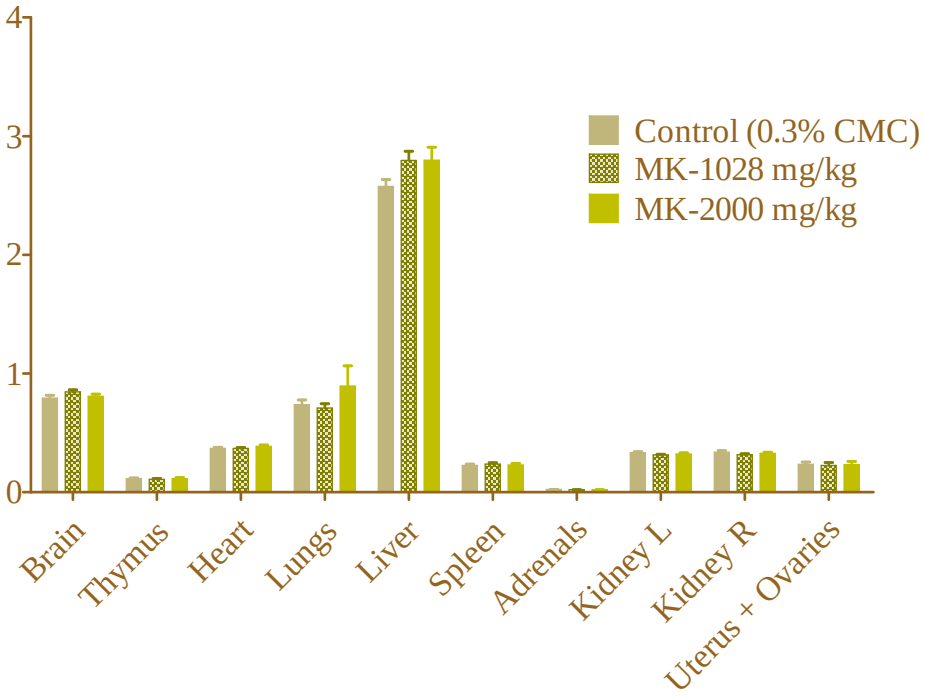
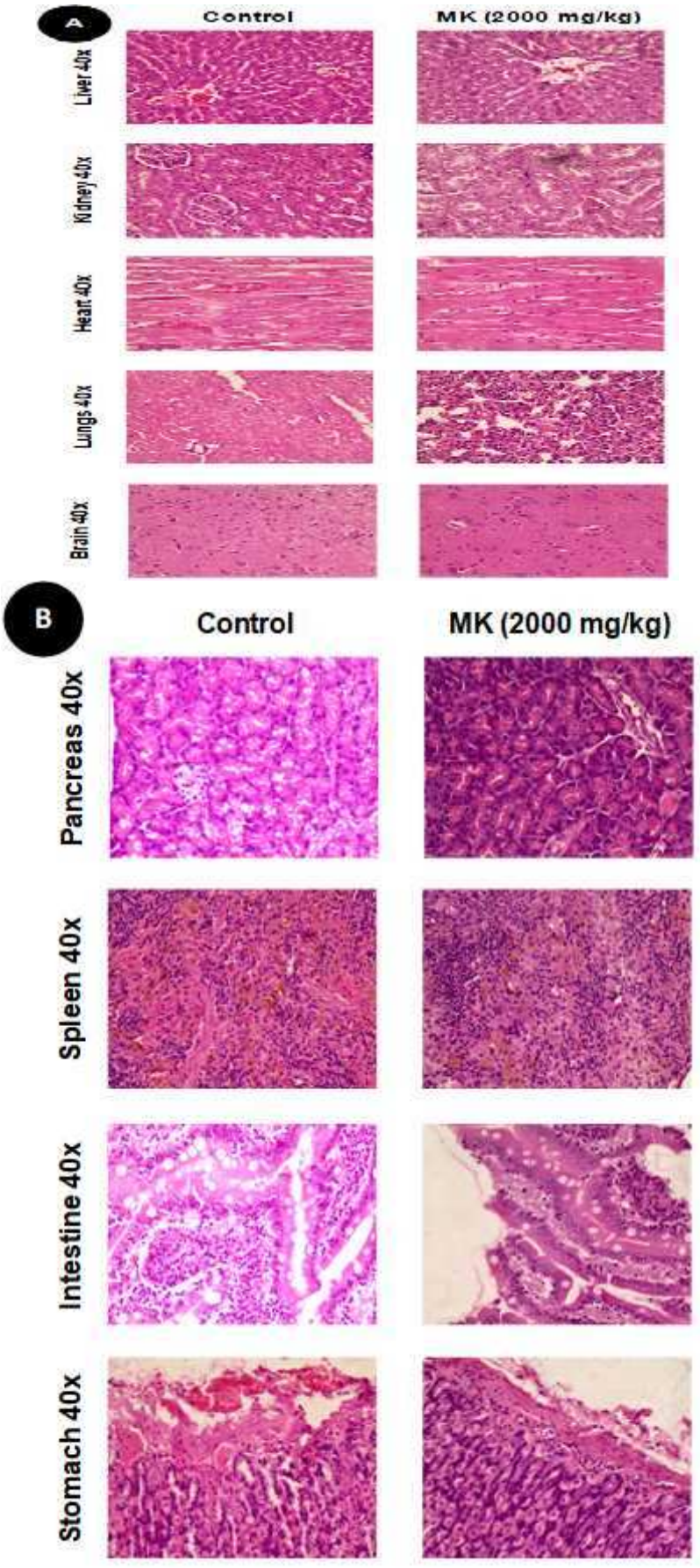


Figure 3 (B): Effect of MK on relative organ weights of female rats


A
Control
MK (2000 mg/kg)
Liver 40x
Kidney 40x
Heart 40x
Lungs 40x
Brain 40x
B
Control
MK (2000 mg/kg)
Pancreas 40x
Spleen 40x
Intestine 40x
Stomach 40x

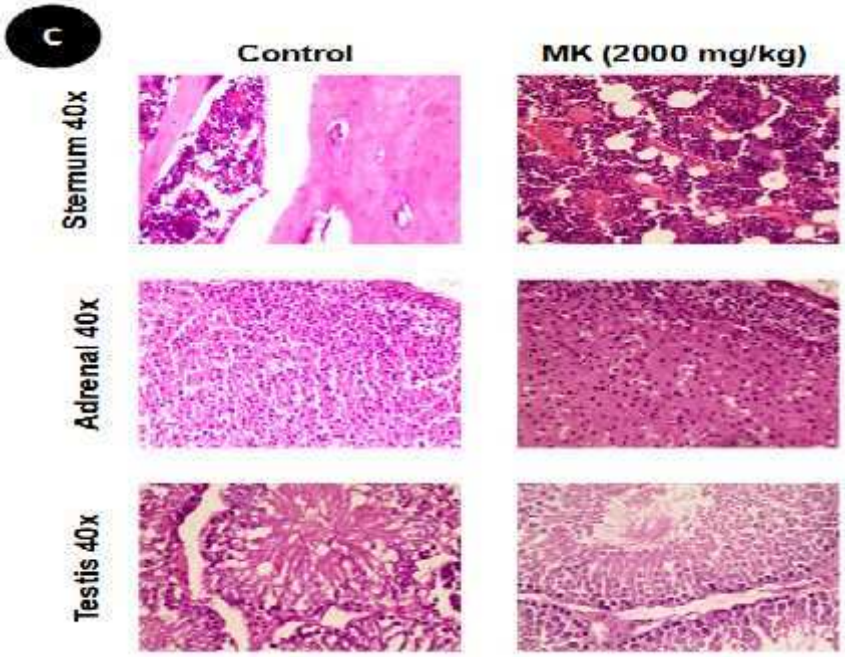


Figure 4 A-C: Representative photomicrographs of histological section of Control and MK (2000 mg/kg) treated rats showing normal architecture of different tissues (H&E stain)

Results And Discussion

MK has been used for decades in the Unani system of medicine. Although this classical formulation is effective in various disease conditions but no scientific report is available for its safety on prolonged use. This study evaluated long term effects on MK following repeated dose oral administration for 90 days in SD rats. Rats of control group and groups treated with MK were subjected to clinical examination at different time points. Both groups did not show any abnormal behaviour or clinical signs indicative of systemic toxicity. All the rats in the study survived throughout the 90-days study period. Body weight gain in control and MK treated groups did not show any significant difference throughout the study [Figure 1(A) and 1(B)]. There was no statistically significant difference noted in the feed intake of MK treated group in any sex compared to the vehicle treated control rats [Figure 2(A) and 2 (B)]. Body weight gain and feed consumption are non-specific, broad screen for adverse systemic toxicity [7]. The consistent observation of normal and expected pattern in body weight gain and feed consumption dynamics of MK-treated rats of both sexes throughout the dosing period suggested normal growth and development pattern.The haemopoietic system is the primary target of many xenobiotics and is a sensitive indicator for pathological conditions [8]. Haemglobin level of MK treated groups showed no significant difference compared to control group at both 1028 and 2000 mg/kg dose levels in both male and female rats (Table 1). RBC count was significantly low only in males at high dose ($P<0.001$) and haematocrit level was significantly decreased in high dose groups ($P<0.01$ for males and $P<0.05$ for females). However, these values were within the normal physiological range and do not carry toxicological relevance. The platelet count in MK treated rats was comparable to control. WBC count was significantly decreased ($P<0.001$) in male rats treated with MK at 1028 and 2000 mg/kg, whereas it was significantly increased ($P<0.01$) in female rats treated with MK at 1028 mg/kg. Notwithstanding, as observed previously, all the values were within the reference range and therefore may not require any toxicological consideration. Female rats which were administered 1028 and 2000 mg/kg doses of MK showed significant reduction in neutrophil count ($P<0.001$) of which group treated with 2000 mg/kg also showed significant increase in lymphocyte count. Male rats also showed significant increase in Eosinophil count at 1028 ($P<0.001$) and 2000 mg/kg ($P<0.01$) doses. Monocyte count was also increased only in males in both lower dose ($P<0.05$) and higher dose ($P<0.001$) groups. In the present study, no alteration in hematological profile with MK treatment indicates no adverse effect of treatment on hemopoiesis.Serum glucose levels in all treatment group showed insignificant difference compared to control group in case of both male and female treatments (Table 2). The glucose levels were also within the normal reference range indicating normal glucose utilisation and glucose tolerance in all treatment groups.Hepatic toxicity is one of the common adverse effect of many clinically used agents leading to restricted use and even withdrawal of drug post marketing [9, 10]. Drug induced hepatic toxicity is characterized by alteration in SGOT, SGPT, ALP, and bilirubin levels [11]. Any elevation pertaining to SGOT and SGPT indicates their outflow into the bloodstream owing to damage in liver parenchymal

cells [12, 13]. In the present study, SGPT levels were significantly reduced in male animal group treated with MK at the dose of 2000 mg/kg (P<0.01). Similarly, ALP was also significantly reduced only in male rats which were administered MK at 2000 mg/kg. No statistically significant difference was observed in SGOT level in any sex at either dose level. Decrease in the levels of SGPT and ALP in males of high dose group may not be considered toxicologically relevant as the values remain in the physiological range. Albumin synthesis is one of the functions of liver, thus, decreased albumin level has a positive correlation with prognosis of liver disease. Bilirubin occurs in both conjugated (glucuronide) as well as unconjugated form. Balance between conjugate and unconjugated form is a measure of reflects balance between production and hepatobiliary excretion. Higher than normal bilirubin level is observed in haemolysis, altered erythropoiesis as well as in muscular injury. Further, conjugated hyperbilirubinaemia signifies liver disease and biliary obstruction [14]. In the present study, control as well as MK treated groups exhibited normal values of albumin and total bilirubin. Serum globulin levels were increased significantly in female groups at both 1028 (P<0.001) and 2000 mg/kg (P<0.01) dose levels but the values were within the normal reference range and hence may not have any toxicological significance. No significant change was observed in serum globulin levels of male rats. The total protein level was also significantly increased in female rats treated with MK at 1028 mg/kg dose (P<0.001). The values, however, were within the normal reference range and the similar increase was not observed in higher dose group of female rats. Therefore, this effect cannot be considered toxicologically significant. Kidneys are the major organ for detoxification and drug excretion and are one of the main targets for toxic effects of drug similar to liver. Serum creatinine and BUN are the standard indicators of renal function in preclinical or clinical trials and increased creatinine and BUN levels are indicator of altered renal function [15]. BUN levels were significantly decreased in male MK treated rats at the dose of 1028 mg/kg (P<0.05) as well as in female rats treated at both 1028 (P<0.001) and 2000 mg/kg (P<0.01) dose levels with respective control. Further, no significant changes were observed in the serum creatinine levels of either sex at any dose level of MK. There was significant increase in cholesterol (P<0.05) and LDL (P<0.001) levels in male groups at the lower dose level compared to control. However, the similar increase was not observed in higher *i.e.* 2000 mg/kg treated groups. Thus, the effect may not be considered treatment related or to have any significance from toxicological perspective. Further the values in both the case was within the normal reference range. Further, there was a significant reduction in serum triglyceride level (P<0.01) as well as serum VLDL levels (P<0.01) in the male groups treated with MK at 1028 mg/kg. However, the values for cholesterol, triglycerides, LDL as well as VLDL were within normal reference range, therefore, the changes may not carry toxicological significance. Cholesterol/HDL ratio was significantly increased (P<0.01) in male groups treated with both 1028 and 2000 mg/kg doses of MK. Similarly LDL/HDL ratio was also increased in these group at both 1028 (P<0.001) as well as 2000 mg/kg (P<0.05) dose levels. However, there were insignificant changes in cholesterol/HDL ratio as well as LDL/HDL ratio of female control and MK treated groups.Normal serum electrolyte levels are required to maintain physiological activities. Sodium and potassium are the most abundant extracellular ions which are necessary for normal muscle contraction. The deranged levels of sodium and potassium resulting from the reduced serum level can lead to arrhythmia, abdominal pain and cramping, and muscle weakness [16]. Serum sodium levels in male MK treated rats did not show any significant difference compared to control group, however, female MK rats showed significant increase in serum sodium levels (P<0.001) (Table 3). Serum potassium and chloride level in all treatment groups were within the reference range and did not show and significant change upon treatment with MK at 1028 and 2000 mg/kg dose levels. Serum calcium levels were significantly reduced in both male and female treatment groups upon treatment with MK at 1028 (P<0.001) and 2000 mg/kg (P<0.001) dose levels. However, chronic toxicity data from our laboratory on MK showed no significant difference compared to control in any sex (unpublished data). No gross abnormalities were observed during necropsy in any treatment group including control. The evaluation of organ weights in toxicology studies is an integral component in the assessment of new drugs. The Society of Toxicologic Pathology advocates the routine calculation and evaluation of organ/body weight ratios in toxicology studies lasting from 7 days to one year [17]. Organ/body weight ratios (i.e., relative organ weight) were considered more useful when body weights were affected [18]. Relative organ weights of both male and female rats treated with MK at the two tested dose levels were comparable to control [Figure 3 (A) and 3 (B)]. Representative photomicrographs of histological section of control and high dose MK-treated groups have been presented in Figure 4 A-C. There were significant histological alterations observed only in lungs and liver of experimental animals. However, in both lungs and livers the various histological changes observed in experimental groups were also observed in the control group and hence cannot be attributed to the administration of the MK. All the remaining organs were normal histologically.

Conclusion

90-days repeated dose oral toxicity study was conducted on Majoon Kundur as per OECD guidelines. There were no toxicologically significant observation with respect to clinical signs of toxicity, body weight, feed consumption, haematology, clinical chemistry, organ weight, and gross necropsy findings in MK treated rats at 1028, 2000 mg/kg bw or in control animals. The histopathological finding did not reveal any treatment related toxicologically significant changes. Thus, the No Observed Adverse Effect Level (NOAEL) of MK may be considered >2000 mg/kg bw in rats.

Acknowledgement

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Table 1: Effect of MK on Haematology in Rats

Parameter	Male			Female		
	Control	MK-1028	MK-2000	Control	MK-1028	MK-2000
Hb (gm%)	17.42±0.32	16.58±0.25	16.52±0.30	16.83±0.29	15.98±0.25	15.93±0.26
RBC (Million/mm^3)	9.15±0.08	8.79±0.09	8.64±0.14**	8.64±0.12	8.4±0.09	8.35±0.12
HCT (%)	46.79±0.46	45.66±0.35	44.64±0.50**	46.03±0.54	44.91±0.43	44.36±0.40*
Platelet (lakhs/mm^3)	4.48±0.38	3.94±0.06	4.41±0.23	4.24±0.29	4.35±0.12	4.07±0.37
WBC (/mm^3)	10190±257.5	7390±330.8***	7760±424.6***	8280±285.9	9930±433.1**	8840±336.4
Neutrophil (%)	18.1±1.02	17.4±2.07	20.3±3.31	17.3±0.80	12.1±1.03***	12±0.73***
Lymphocyte (%)	75.9±1.29	72.7±2.18	72.7±2.12	76.7±0.99	80.3±0.95	81.9±1.28**
Eosinophil (%)	3.5±0.52	5.9±0.1***	5.6±0.34**	3.6±0.37	4.6±0.22	4.6±0.27
Monocyte (%)	2.5±0.27	3.7±0.26*	4.4±0.30***	2.4±0.16	3±0.21	2.5±0.34

Values represented as Mean ± SEM; *P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control

Table 2: Effect of MK on Blood Biochemical Parameters in Rats

Parameter	Male			Female		
	Control	MK-1028	MK-2000	Control	MK-1028	MK-2000
Glucose (mg/dL)	94.4±4.58	107.5±8.40	87.8±3.56	89.8±1.65	82.6±5.04	81.7±5.40
SGPT (IU/L)	86.7±3.03	72.5±6.93	64.8±3.24**	77.4±5.04	77.2±10.2	71.6±4.53
SGOT (IU/L)	137.8±8.33	136.4±9.74	113.8±3.35	125.8±4.99	114.5±12.12	131.9±5.02
BILIRUBIN (mg/dL)	0.131±0.009	0.229±0.075	0.128±0.009	0.171±0.008	0.16±0.009	0.144±0.006
ALP (IU/L)	115.7±3.67	109±5.18	87.1±7.41**	93.2±9.72	102.6±6.72	89.7±4.26
TP (g/dL)	6.37±0.07	6.53±0.10	6.44±0.12	5.97±0.06	7.04±0.16***	6.38±0.13
Albumin (g/dL)	4.09±0.11	4±0.05	3.8±0.10	4.06±0.09	4.23±0.07	3.91±0.06
Globulin (g/dL)	2.28±0.098	2.53±0.093	2.64±0.180	1.71±0.146	2.79±0.134***	2.47±0.123**
AG ratio	1.81±0.119	1.48±0.076	1.456±0.118	2.56±0.295	1.446±0.010	1.57±0.092
BUN (mg/dL)	16.64±0.66	12.47±0.95*	13.9±1.25	18.53±0.72	13.34±0.85***	11.13±0.51**
Creatinine (mg/dL)	0.88±0.021	0.94±0.027	0.92±0.013	0.85±0.022	0.89±0.0180	0.92±0.029
Cholesterol (mg/dL)	79.6±4.001	100±5.554*	86.9±5.561	115.9±4.428	129.8±5.555	127.5±4.235
TGs (mg/dL)	61.2±3.09	46.7±2.381**	62.5±3.787	48.6±2.358	49.4±3.219	52.2±5.048
HDL (mg/dL)	47.2±1.009	42.4±1.343	38.8±2.102**	52.5±2.729	50.7±1.82	57.7±2.868
LDL (mg/dL)	20.1±3.24	48.2±4.32***	35.7±5.13*	53.7±3.31	69±4.96*	59.4±3.88
VLDL (mg/dL)	12.3±0.66	9.4±0.50**	12.4±0.75	9.7±0.47	10.1±0.63	8.78±1.68
Chol/HDL	1.683±0.074	2.3±0.076**	2.22±0.166**	2.231±0.090	2.53±0.128	2.2±0.107
LDL/HDL	0.42±0.065	1.07±0.078***	0.899±0.156*	1.046±0.082	1.34±0.119	1.01±0.089

Values represented as Mean ± SEM; *P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control

Table 3: Effect of MK on serum electrolyte level in Rats

Parameter	Male			Female		
	Control	MK-1028	MK-2000	Control	MK-1028	MK-2000
Sodium (mmol/L)	137.3±0.5	135.4±0.8	139.3±1.5	137.4±0.3	144.3±0.5***	147.5±0.4***
Potassium (mmol/L)	4.55±0.06	4.28±0.13	4.29±0.06	4.39±0.07	4.22±0.11	4.21±0.07
Chloride (mmol/L)	101.2±0.6	104.2±0.8	98.4±1.5	104.7±0.5	104±0.5	106.4±0.6
Calcium (mmol/L)	6.79±0.45	2.37±0.12***	1.9±0.09***	5.57±0.41	1.24±0.02***	0.84±0.03***

Values represented as Mean ± SEM; ***P<0.001 vs Control